PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/10 // A61K 39/00
C07K 15/00

A1

(11) International Publication Number: WO 93/01285

(43) International Publication Date: 21 January 1993 (21.01.93)

(21) International Application Number: PCT/GB92/01231

(22) International Filing Date: 7 July 1992 (07.07.92)

(30) Priority data:
9114734.8 9 July 1991 (09.07.91) GB

(71) Applicant *(for all designated States except US)*: UNIVERSITY COLLEGE LONDON [GB/GB]; Gower Street, London WC1E 6BT (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : DELVES, Peter, John [GB/GB]; 105 Woodlands Avenue, London E11 3RB (GB). LUND, Torben [DK/GB]; 45 Lyttleton Court, Lyttleton Road, London N2 0EB (GB). ROITT, Ivan, Maurice [GB/GB]; 995 Finchley Road, London NW11 7HB (GB).

(74) Agent: EYLES, Winifred, Joyce; Patents Department, British Technology Group Ltd., 101 Newington Causeway, London SE1 6BU (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: PROCESS FOR MODIFYING PROTEINS BY RANDOM MUTATION

(57) Abstract

A process is described for the modification of a protein product of recombinant DNA technology comprising subjecting DNA coding for the protein product, or coding for at least that portion of the protein product to be modified, to random mutation in the presence of at least one chemical or physical mutagenising agent, expression of the mutated DNA in a host organism and screening of the expression products with at least one antibody so as to select the expression product containing the desired modification.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

PROCESS FOR MODIFYING PROTEINS BY RANDOM MUTATION

This invention relates to the preparation of modified proteins and is particularly concerned with the modification of proteins obtained by recombinant DNA technology.

Proteins and polypeptides produced by recombinant DNA technology have in recent years found wide applicability in many areas of vaccine technology, therapy, diagnostics and enzymology. Such synthetically created proteins rely for their effectiveness on the inclusion of critical regions of activity, such as epitopes or binding sites, of which one wishes to make optimum use, but they generally contain other regions of a less critical nature or which are positively harmful to the intended use of the protein.

Thus, for example, if such a protein is intended for use as a vaccine, while one must conserve the epitope or epitopes capable of eliciting the desired immune response, it is in many cases desirable to eliminate other epitopes which hinder the potential usefulness of the vaccine. Such a situation occurs, for example, where an unwanted epitope cross-reacts with a self-molecule and is potentially capable of giving rise to autoantibodies when used for immunization, a situation illustrated by the known streptococcus cross-reaction with heart tissue, giving rise to rheumatic fever. As a further example, it can also be desirable to remove immunodominant epitopes which might otherwise prevent or diminish a protective immune response to other epitopes on the antigen of interest (e.g. HIV gp 120).

One solution to such a problem is to selectively mutate the gene which codes for the protein of interest. However, this approach requires knowledge of the precise sequence of amino acids contributing to the epitope so that the specific corresponding gene sequence can be mutated. As the majority of epitopes recognised by antibodies are discontinuous and rely on their tertiary structure for their effectiveness, such an approach is of very limited application.

An alternative approach is provided by EP-A-341444 which aims to identify and characterise epitopes of cell surface proteins such as CD2 and CD4 by first preparing a series of oligonucleotides, covering the substitution of all possible base pairs encoding the extracellular domain of the protein under investigation, separate transformation of each of the oligonucleotide-modified recombinant plasmids in E. coli to yield a series of primary mutants which were used to transfect mammalian cells, followed by selection of the desired expression product. The method is complex and applicable only to cell surface proteins. Winter and Milstein (Nature, 349, 1991, 293-299) propose in vitro imitation of antibody "hypermutation" by site specific genetic engineering techniques. Such methods are comparatively complicated and are directed against predetermined restricted sites in the molecule.

There is therefore clearly a need for a straightforward means whereby a wide range of protein products (e.g. whole native proteins or portions thereof) of recombinant DNA technology can be tailored to make them better adapted to the task for which they are intended.

Accordingly the present invention provides a process for the modification of a protein product of recombinant DNA technology comprising subjecting DNA (suitably cDNA or genomic DNA) coding for the protein product, or coding for at least that part of the protein product to be modified, to random mutation in the presence of at least one chemical or physical mutagenising agent, expression of the mutated DNA in a host organism and screening of the expression products with at least one antibody so as to select the expression product containing the desired modification.

When the random mutation is carried out on DNA coding for less than the complete protein product, for example when coding only for the specific portion of the protein product to be modified, it will be necessary in order to obtain the desired modified protein product to employ genetic engineering

techniques to introduce the remaining protein product gene sequence or sequences following the random mutation. Such introduction may be before expression of the DNA in the host organism or even downstream of the screening procedure.

The invention also includes modified proteins obtained by this process and vectors such as plasmids capable of expressing the modified proteins in a host organism.

The starting DNA corresponding to the protein product to be modified may be obtained by standard genetic engineering techniques including screening of cDNA and genomic libraries with oligonucleotide probes based on partial amino acid sequence, or screening of expression libraries of PCR-amplified DNA using antibodies. The DNA is suitably incorporated in a vector, suitably a carrier plasmid prior to the random mutation step, the plasmid selected being dependent on the DNA sequence to be ligated therewith and the host organism in which the DNA is to be expressed.

The modification desired may be the deletion of an unwanted immunoactive epitope or binding site, in which case the screening of the expression products comprises a two stage selection which involves detecting with a suitable monoclonal antibody the absence of the unwanted site and detecting with a suitable mono- and/or polyclonal antibody the presence of the activity desired in the modified protein product. Alternatively, if a modification such as enhancement of an active site of the protein product is desired, the screening may comprise a single selection step employing a reagent reacting with the active site, such as a ligand, a receptor, or an enzyme substrate.

It will be appreciated that the process of the invention allows one to tailor the protein itself to retain or enhance the epitope of interest while overcoming problems resulting from unwanted sites in the protein. The invention therefore exploits the unique ability of proteins to fold and form epitopes (as

distinct from synthetic peptides) but allows one to eliminate unwanted features of the proteins.

The chemical or physical mutagenising agent may be any such agent that is suitable for effecting random mutations in a nucleotide sequence. One suitable example of a chemical agent is hydroxylamine (which is known to induce AT to GC transitions and vice versa) and which can cause mutation directly of a plasmid containing the desired DNA sequence in the absence of a host in which the plasmid is expressed. A typical strategy for use with hydroxylamine as mutagenising agent comprises incubating plasmid containing the cDNA corresponding to the protein to be modified with hydroxylamine (suitably as its hydrochloride) at a concentration ranging from 0.5 to 2M at 37°C for a time period from 8 to 24 hours, preferably about 18 hours. The DNA is precipitated and the mixed mutated and unmutated plasmids transformed in E. coli grown for a few generations, followed by plating onto the growth media to obtain single colonies, blotting onto a medium such as nitrocellulose and subsequent screening of the expressed proteins with appropriate antibodies. The desired mutated plasmid DNA is recovered from the corresponding bacterial colony and can be used to express the desired modified protein.

An alternative chemical mutagenising agent is N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which is known to induce transitions and transversions, together with small deletions, at a low rate in the presence of a host in which the plasmid is expressed. A typical strategy for use with MNNG as mutagenising agent comprises growing a host such as E. coli transformed with a plasmid containing the cDNA corresponding to the protein to be modified, exposing an aliquot of recombinant plasmid-containing bacteria to MNNG in a buffer such as sodium acetate, removal of aliquots at suitable time intervals (for example up to 20 minutes), growth of the individual aliquots through several generations, plating onto growth media-containing agar plates to obtain single colonies and application of suitable selection

procedures as for hydroxylamine mutagenesis. Other possible agents include O-methyl hydroxylamine, nitrous acid and ethyl methane sulphonate, or DNA polymerase used in a polymerase chain reaction (PCR) method. Alternatively, ultra violet irradiation may be used as a physical mutagenising agent.

As described above, the products of mutagenesis (i.e. a mixture of mutated and unmutated plasmids) are expressed in a suitable host. The host may be a well understood bacterial host such as E. coli K12, in which case a known plasmid such as pEX2 (described by Stanley and Luzio, 1984, EMBO J., 3, 1429), or any other vector which allows the expression of cloned DNA in a host such as E. coli, may be employed to carry the DNA of interest. However, it is also contemplated that expression may be carried out in other host systems such as yeast cells and mammalian cells.

As described above, the screening of the expression products depends on whether it is desired to produce an "epitope loss" protein product or a "locally modified" protein product. For screening to obtain a desired epitope loss product, following expression for example in E. coli, the bacterial colonies are separated and examined with at least two different antibodies to identify the epitope loss mutants. Those colonies which fail to react with a monoclonal antibody to the epitope to be removed are deemed to have lost that epitope, while ability to react with a polyclonal antiserum and/or a monoclonal antibody to the epitope it is desired to retain is evidence that the desired antigenic structure of the protein has remained intact.

For screening to obtain a "locally modified" product, for example to improve the affinity of an antibody combining site for a particular antigen, a single screening can be employed, using for example a low concentration of antigen and determining those colonies which give an expression product with a high affinity reaction.

It will be appreciated that any convenient analytical technique known in the art can be employed in the screening

procedures, such as expression screening, enzyme-linked immunosorbent assay, Western blotting etc. Where two different antibody reagents are employed, they can each be separately labelled with distinct fluorophors and used as a single mixture, the fluorescence emissions being analysed by appropriate filters.

The above described process is simple and believed to be applicable to any protein produced by recombinant DNA technology without the necessity of knowing the exact sequence or position of the epitopes or local structures to be removed, retained or modified. Iterative mutations can be carried out to sequentially improve upon the desired modification.

The process of the invention is intended for a wide variety of uses. These uses include destroying unwanted epitopes in immunogens being investigated as potential vaccines, e.g. bacterial surface antigens, toxins (for toxoid production), parasites, viruses and also contraceptive vaccines based upon modified immunogenic hormone. Further uses include improving antibody affinity, both for intact monoclonal antibodies and genetically engineered (for example single domain) antibodies. This is particularly relevant to human monoclonal antibodies which are difficult to obtain with high affinity. The process of the invention is contemplated for use in improving the efficiency of catalytic antibodies (known as "abzymes") by randomly altering the protein structure and selecting variants with enhanced function or altered specificity, as well as use in refining the specificity of conventional enzymes. Another application is for the identification of amino acids contributing to the active site of epitopes, hormones and cytokines by further sequencing procedures, and use is also contemplated in the development of hormone and cytokine antagonists and antagonists for cell adhesion molecules made by mutating natural ligands, for example for use as novel anti-inflammatory agents. Use of the process is also contemplated as a powerful technique in the identification of

contact residues of discontinuous B cell epitopes and protein hormones.

The invention will now be described by way of example with reference to the preparation of epitope loss mutants of the bacterial β-galactosidase protein which have been found no longer to react with a given monoclonal antibody but to retain the ability to bind to polyclonal antisera against the native molecule.

Example

A plasmid pEX2 containing the lacZ gene (encoding β-galactosidase) having the structure shown in Figure 1 was utilised (Stanley and Luzio, 1984, EMBO J., 3, 1429).

pEX2 is a plasmid about 5.8kb in length, designed for expression of cDNA fused at the 3' terminus of the lacZ gene. The amino-terminal part of the lacZ gene has been replaced with some sequences from the bacteriophage λ cro gene and the E. coli lacI gene. The bacteriophage λ p_R promoter is used, which allows expression of the fusion protein to be regulated by the bacteriophage λ cIts857 repressor. A polycloning site is present at the 3' terminus of the lacZ gene, followed by the translation stop codons (Stop) and a transcription terminator (Term) from the bacteriophage fd.

The plasmid was randomly mutated with hydroxylamine hydrochloride (HA) using the following procedure. Twenty μg samples of plasmid DNA in 100μl 10mM Tris 1mM EDTA pH 8.0 (TE) were incubated with 2M, 1.5M, 1M or 0.5M HA at 37°C for 18 hrs. After the addition of 0.3M sodium acetate the DNA was precipitated with 2.5 volumes ethanol. Following resuspension in 50μl TE an aliquot of the mutated (and control unmutated) plasmids were inserted into competent E. coli DH5α. The bacteria were then grown for 16 hrs. in order to replicate mutations into both strands of the DNA. Plasmid was then isolated from these bacteria and again used to transform competent DH5α using a ratio of DNA to bacteria which should give only one plasmid per bacterial cell. Transformed cells

were spread onto L-Broth (10g/l Bacto-tryptone, 5g/l yeast extract, 10g/l sodium chloride) agar plates containing 50µg/ml ampicillin. After growth at 30°C the colonies were 'lifted' onto nitrocellulose membranes and the filters duplicated. The filters were then grown on agar plates for 2 hrs. at 42° to induce protein expression. Control (uninduced) filters were maintained at 30°C.

Bacteria on the filters were lysed by incubation for 25 min. at 95°C on 3MM paper soaked in 5% sodium dodecyl sulphate (SDS). The SDS residue was then removed and the protein fixed to the nitrocellulose membrane by electrophoresis for 1 hr. (buffer 0.2M Tris base containing 1.6M glycine). Filters were washed three times in 0.1% Triton X-100, 0.5% gelatin in phosphate buffered saline for 5 min. each, the second wash additionally containing 10µg/ml DNAse.

Prior to reaction with antibodies, the filters were washed in TNT (10mM Tris pH 8.0 150mM NaCl; 0.05% Tween 20) for 20 min. and then transferred to 3% bovine serum albumin (BSA) in TNT in order to block remaining protein binding sites on the nitrocellulose. Filters were incubated with pre-determined optimal dilutions of primary antibody (1:1000 - 1:5000 of mouse monoclonal or rabbit polyclonal anti-β-galactosidase) for 150 min. at 20°C in blocking buffer and then washed three times for 10 min. in 0.1% BSA in TNT. The middle wash buffer also contained 0.1% Nonidet P40 (detergent supplied by BDH). Filters were then incubated for 90 min. at 20°C with a 1:2000 dilution of alkaline phosphatase- conjugated goat anti-mouse IgG or anti-rabbit IgG antibody followed by washing as previously. Reactivity of the antibodies was detected by incubation of the filters with nitroblue tetrazolium (NBT)/ 5-bromo-4-chloro-3 indolyl phosphate (BCIP) in the dark for 45 min. followed by two washes in double distilled water.

Confirmation of specificity was obtained by the inclusion of irrelevant antibodies such as anti-thyroglobulin. Furthermore, the protein product of the bacteria was analysed by SDS-

polyacrylamide gel electrophoresis followed by Western blotting using anti-β-galactosidase antibodies.

There were clearly identified plasmids which no longer had the ability to express protein reacting with the monoclonal antibody and had therefore lost the corresponding epitope, while those same proteins retained the ability to react with anti-β-galactosidase polyclonal antibodies.

CLAIMS

1. A process for the modification of a protein product of recombinant DNA technology comprising subjecting DNA coding for the protein product, or coding for at least that portion of the protein product to be modified, to random mutation in the presence of at least one chemical or physical mutagenising agent, expression of the mutated DNA in a host organism and screening of the expression products with at least one antibody so as to select the expression product containing the desired modification.

2. A process according to claim 1 wherein the DNA subjected to random mutation is cDNA.

3. A process according to claim 1 wherein the DNA subjected to random mutation is genomic DNA.

4. A process according to any one of claims 1 to 3 wherein the DNA codes for the entire protein product.

5. A process according to any one of claims 1 to 3 wherein the DNA codes for less than the complete protein product and the remaining protein product gene sequence or sequences are introduced following the random mutation.

6. A process according to any one of the preceding claims wherein the DNA is incorporated in a carrier plasmid prior to mutation.

7. A process according to any one of the preceding claims wherein the host organism is E. coli.

8. A process according to any one of the preceding claims wherein the mutagenising agent is hydroxylamine.

9. A process according to any one of claims 1 to 3 wherein the mutagenising agent is N-methyl-N'-nitro-N-nitrosoguanidine.

10. A process according to any one of claims 1 to 7 wherein the mutagenising agent is ultraviolet irradiation.

11. A process according to any one of the preceding claims wherein the modification desired is epitope loss and the screening comprises selection procedures comprising identifying modified proteins which do not react with a monoclonal antibody

to the epitope whose loss is desired but do react with a polyclonal and/or monoclonal antibody to the epitope to be retained in the modified protein.

12. A modified protein when obtained by the process of any one of the preceding claims.

13. A vector comprising a mutated DNA coding for a modified protein obtainable by the process of any one of claims 1 to 12.

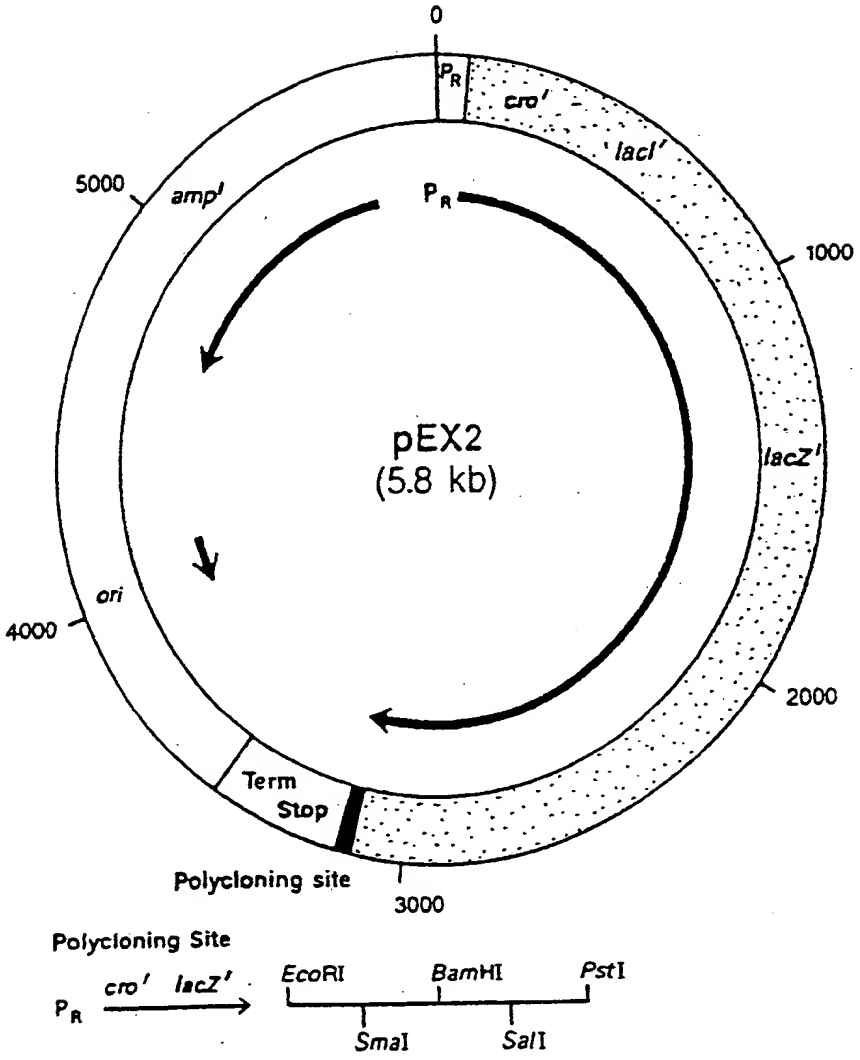
0
P_R
cro'
'lacI'
1000
2000
3000
4000
5000
amp'
ori
pEX2
(5.8 kb)
P_R
lacZ'
Term
Stop
Polycloning site
Polycloning Site
P_R
cro' lacZ'
EcoRI
BamHI
PstI
SmaI
SalI

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01231

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/10; //A61K39/00,C07K15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	J. SAMBROOK ET AL 'Molecular cloning. A laboratory manual' 1989 , COLD SPRING HARBOR LABORATORY PRESS	1-10,12, 13
Y	see page 15.105	11
X	US,A,4 894 331 (B.J. RATZKIN ET AL) 16 January 1990	1-10,12
Y	see abstract; example 1	11
X	WO,A,8 705 050 (GENEX CORPORATION) 27 August 1987	1-10,12
Y	see abstract	11
Y	EP,A,0 341 444 (THE GENERAL HOSPITAL CORPORATION) 15 November 1989 cited in the application see page 2, line 40 - line 47	11

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 OCTOBER 1992	19. 10. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 375 889 (DEGUSSA AG) 4 July 1990 see claim 1	1,8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201231 SA 61587

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 01/10/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4894331	16-01-90	None	
WO-A-8705050	27-08-87	EP-A- 0260299	23-03-88
		JP-T- 63502959	02-11-88
		US-A- 4980288	25-12-90
		US-A- 4990452	05-02-91
EP-A-0341444	15-11-89	JP-A- 2203787	13-08-90
EP-A-0375889	04-07-90	DE-A- 3841454	13-06-90
		AU-A- 4608589	14-06-90